

## RAPID IDENTIFICATION OF MYCOBACTERIUM TUBERCULOSIS COMPLEX BY A NOVEL HYBRIDIZATION SIGNAL AMPLIFICATION METHOD BASED ON SELF-ASSEMBLY OF DNA-STREPTAVIDIN NANOPARTICLES

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### ABSTRACT

Rapid detection of *Mycobacterium tuberculosis* complex (MTBC) is a critical step in controlling tuberculosis (TB). In this study, we used IS6110 as the specific identification target to develop a novel hybridization signal amplification method (HSAM) for the rapid and direct detection of MTBC from clinical sputum specimens. This system consists of magnetic bead-linked capture probes for target isolation, dextran-based nanoparticles for amplifying the reporter molecule (biotinylated-FITC), and detection probes (2B-DNA) for binding the nanoparticles. Both the capture and detection probes were specific to the IS6110 target sequence. Our results determined that as few as 10 copies of the IS6110 sequence or 10 *M. tuberculosis* bacteria could be detected, indicating that the HSAM assay is as sensitive as conventional PCR, and the assay was specific enough to distinguish MTBC from nontuberculosis mycobacteria (NTM). A total of 176 clinical sputum specimens were collected for HSAM evaluation, and the results were compared to those from traditional culture and biochemical identification techniques. This assay had a sensitivity of 88.3%, a specificity of 91.8%, a positive predictive value of 93.8% and a negative predictive value of 84.8% for the detection of MTBC. This technique is highly sensitive and specific, is easy to perform, and does not require any sophisticated detection equipment; thus, this approach has great potential in clinical TB detection and diagnostic applications.

**Key words:** *Mycobacterium tuberculosis* complex (MTBC); Detection; Diagnosis; Rapid methods; Identification

### INTRODUCTION

Tuberculosis (TB) is one of the greatest health concerns in developing and industrialized countries, affecting 1.7 billion people worldwide. There are 8 million new cases and around 3

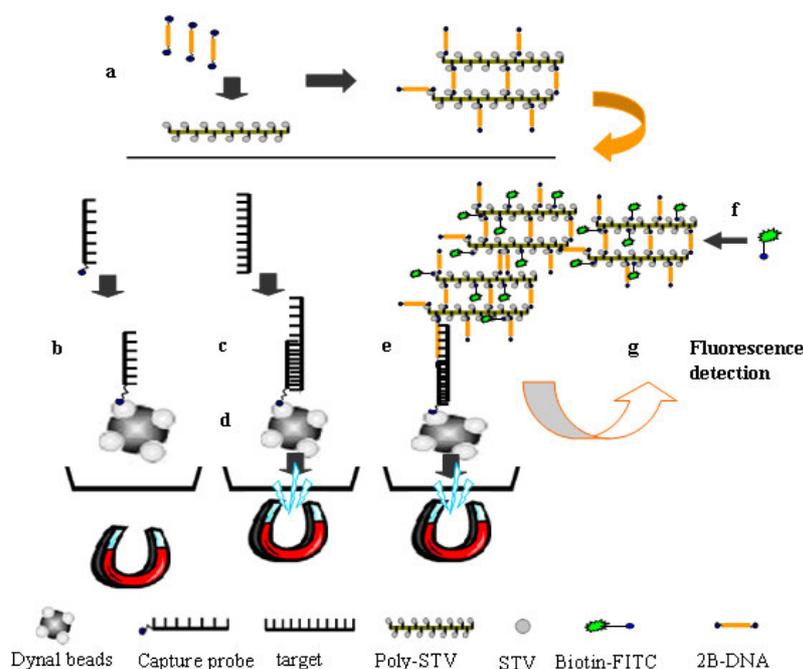
million deaths annually. Alarming, the number of TB cases is rising at a rate of 2% per year. Rapid detection of *Mycobacterium tuberculosis* complex (MTBC) strains is one of the most important factors in minimizing the spread of this disease. In low- and middle-income countries, conventional

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smear microscopy and culture are the primary methods for diagnosis of pulmonary tuberculosis. Smear microscopy is easy, fast and inexpensive, but its efficiency is limited by relatively low sensitivity (3, 15, 17, 19). Cultures can take from 6 to 8 weeks to obtain conclusive results, limiting this technique's ability to aid in diagnosis and inform immediate treatment decisions. Recently, several unique detection systems have been developed as rapid tests for the direct identification of MTBC from clinical specimens, including a hybridization assay on microwell plates, a DNA microarray assay and cycling probe technology (2, 5, 14). Variations in sensitivity and the high costs of these tests have prevented the widespread use of these systems for the detection of TB.

We have recently developed a novel hybridization signal amplification method (HSAM) for the rapid identification of

MTBC from clinical specimens. In this study, we combine magnetic bead-based DNA isolation, amplification of a reporter molecule (biotinylated-FITC) by nanoparticles and fluorescence detection. *IS6110* was selected as a target site for detection (8), and is specifically recognized by the capture and detection probes. The biotinylated capture probe was immobilized on streptavidin-coated Dynal magnetic beads, taking advantage of the high affinity between biotin and streptavidin (STV) (12, 23). The bis-biotinylated detection probe (2B-DNA) was conjugated to DNA-streptavidin nanoparticles (10, 11). Because each nanoparticle provides multiple binding sites for the biotinylated FITC reporter molecule, a thousand-fold amplification can be achieved (Fig. 1). The objective of this study was to determine the sensitivity and specificity of the HSAM assay for MTBC, and to assess its feasibility for the detection of MTBC from clinical specimens.



**Figure 1.** Schematic representation of the HSAM assay based on self-assembly of DNA-streptavidin nanoparticles. (a) Bis-biotinylated DNA detection probes (2B-DNA) and poly-streptavidin (Poly-STV) form a nanoparticle. (b) The biotinylated capture probe was immobilized onto streptavidin-coated Dynal magnetic beads because of the high-affinity interaction of biotin with streptavidin. (c) *IS6110* was selected as a target site for detection. It is specifically recognized by the capture probe and detection probe. (d) The remaining free biotin binding sites of the surface-bound STV on the beads were blocked with d-Biotin, represented by shaded spheres. (e) The 2B-DNA hybridized to target, and immobilized the nanoparticles to the beads. (f) The reporter molecule (biotinylated-FITC) binds to the nanoparticle. (g) The results of the HSAM assay were obtained using fluorescent microscopy.

## MATERIALS AND METHODS

### Bacterial strains

The *Mycobacterium tuberculosis* (MTB) H37Rv strain was provided by the Center for Disease Control, China. To produce samples with a known number of bacteria, *M. tuberculosis* was grown in 7H9 broth medium (4). Several bacterial species were used to evaluate the specificity of the HSAM reaction: *Mycobacterium bovis*; nontuberculosis mycobacteria (NTMs) including *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, *Mycobacterium chelonae* and *Mycobacterium fortuitum*; and other bacteria such as *Escherichia coli*, *Staphylococcus aureus*,  $\beta$ -hemolytic *Streptococcus* and *Streptococcus pneumoniae*.

### HSAM assay

The HSAM assay involved several steps. First, the capture probe was immobilized on the magnetic beads in a 100  $\mu$ l reaction mixture containing 97  $\mu$ l of binding buffer (20 mM Tris-HCl, 1 M LiCl, 2 mM EDTA), 1  $\mu$ l of capture probe (2 nM) and 2  $\mu$ l ( $10^5/\mu$ l) of Dynabeads® M-280 Streptavidin (Invitrogen Dynal AS, California, USA). The mixture was incubated at 37°C for 20 min at 200 rpm to allow the streptavidin-coated beads to bind the biotin on the capture probe. Second, the target sequence was hybridized to the capture probe by adding 2  $\mu$ l of supernatant from a DNA extraction to the reaction mixture and incubating at 55°C for 1 h. Third, the samples were washed by applying a magnetic field for 5 min to attract the magnetic beads with the attached capture probe/target complex to a specific location in the reaction container. The magnetic beads were washed three times with 400  $\mu$ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA) at room temperature to remove unhybridized capture probe and target DNA. Fourth, the free biotin-binding sites were blocked by adding 100  $\mu$ l of blocking buffer (10 mM Tris-HCl, 1 mM EDTA, 800  $\mu$ M d-Biotin) to the magnetic bead pellet and incubating at 37°C for 20 min at 200 rpm (11). This process blocked the unoccupied biotin-binding sites of the

surface-bound STV on the beads. The samples were then washed three times with 400  $\mu$ l of TE buffer. Fifth, the nanoparticles were immobilized by adding 95  $\mu$ l of binding buffer and 5  $\mu$ l of streptavidin-DNA nanoparticles to the reaction mixture. This mixture was incubated at 55°C for 1 h to allow the complete hybridization of 2B-DNA to the target. The samples were then washed three times with 400  $\mu$ l of TE buffer to remove unhybridized nanoparticles. Sixth, the read-out of the HSAM assay was obtained by adding 40  $\mu$ l of TE buffer and 1  $\mu$ l (2  $\mu$ M) of biotinylated FITC to the mixture and incubating the samples at 37°C for 20 min at 200 rpm. The samples were washed three times with 400  $\mu$ l of TE buffer to remove unhybridized FITC. Finally, the results of the HSAM assay were obtained using fluorescent microscopy (Olympus Optical Co., Ltd, Tokyo, Japan).

### Specimen collection and processing

A total of 176 clinical sputum specimens were collected from patients who presented with clinically and radiologically suspected pulmonary tuberculosis at the Mycobacteriology Laboratory, Hospital of Tuberculosis, Changchun, China between August 2006 and March 2007. The specimens were decontaminated by the addition of an equal volume of sputolysin-NaOH (4%) and were incubated for 20 min at room temperature while rocking. After neutralization with 0.067 M sodium phosphate buffer, pH 5.3 (7, 25), the mixture was centrifuged at 2800 $\times$ g for 20 min. The supernatant was discarded, and the pellets were resuspended in 1 ml of phosphate buffered saline (PBS, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 0.137 M NaCl, 2.7 mM KCl, pH 7.4).

### Culture and biochemical methods for diagnosis of MTBC

About half of each pellet containing suspected pulmonary tuberculosis specimens was inoculated into two Lowenstein-Jensen agar slants and incubated at 37°C with 5% CO<sub>2</sub>. Mycobacterial growth was observed over 2-8 weeks after inoculation. Positive cultures for acid-fast bacilli were

identified by conventional biochemical tests (8, 16).

### Sample preparation for HSAM

To prepare a sample for HSAM evaluation, 500  $\mu$ l of diluted bacterial culture or sputum pellet was centrifuged at 2800 $\times$ g for 20 min. The supernatant was removed, and the pellets were resuspended in 500  $\mu$ l of distilled water. The mixture was vortexed with glass beads and boiled at 100°C for 10 min (6). This process was repeated three times, and then the mixture was centrifuged at 8000 $\times$ g for 5 min. The resulting supernatant was used in the HSAM assay.

### Probe preparation

The detection (2B-DNA) and capture probes were derived from the nucleic acid sequences encoding the insertion sequence IS6110 (GenBank M29899). The capture probe was derived from nucleic acids at positions 431 to 470 with a biotin label on the 5' end. The detection probe was derived from positions 471 to 530 with the 5' and 3' ends labeled with biotin (TaKaRa Biotechnology (Dalian) Co., Ltd, Dalian, China). IS6110 was selected as a target site that was specifically recognized by the capture and detection probes (Table 1).

**Table 1.** The nucleotide sequence of synthetic target, capture probe, and 2B-DNA.

| Probe (no. of nucleotides) | Sequence (5'-3')*   |
|----------------------------|---|
| Target (100)               | <i><u>TTTGCGGTGGGGTGTGCGAGTCGATCTGCACACAGCTGACC</u></i><br><i><u>GAGCTGGGTGTGCCGATCGCCCCATCGACCTACTACGACC</u></i><br><i><u>ACATCAACCGGGAGCCCAGC</u></i> |
| Capture probe (44)         | Biotin-ggaaGGTCAGCTGTGTGCAGATCGACTCGACACCCCA<br>CCGCAAA   |
| 2B-DNA (60)                | Biotin-aagaGCTGGGCTCCCGTTGATGTGGTCGTA<br>GTAGGTCGATGGGGCGATCGGCACACCCA-Biotin   |

\*The singly underlined nucleotides indicate that the sequence is complementary to 2B-DNA; italic and bold nucleotides indicate a sequence that is complementary to the capture probe; and doubly underlined nucleotides represent non-complementary nucleotides to 2B-DNA and the capture probe. Lowercase letters represent non-complementary nucleotides to the target.

### Nanoparticle assembly

Dextran-based poly-streptavidin (Poly-STV, 110  $\mu$ M) (Dako A/S, California, USA) and 2B-DNA were diluted to 5  $\mu$ M and 1  $\mu$ M, respectively, with buffer B (1 M Tris-HCl pH 7.5, 0.5 M EDTA, pH 8.0). Nanoparticles were assembled in 30  $\mu$ l reaction mixtures in the presence of 15  $\mu$ l Poly-STV (5  $\mu$ M) and 15  $\mu$ l 2B-DNA (1  $\mu$ M). The reaction mixture was incubated at 55°C for 30 min at 800 rpm. The products were analyzed by gel electrophoresis using a 1.0% agarose gel and atomic force microscopy.

### Validation of HSAM

To evaluate the sensitivity of the test, ten-fold serial dilutions of the synthetic IS6110 DNA target and *M. tuberculosis* H37Rv were prepared. A culture of *M.*

*tuberculosis* H37Rv grown in 7H9 broth medium was adjusted to the turbidity equivalent of the No. 4 McFarland barium sulfate nephelometer standard (22). Approximately 10<sup>5</sup> colony-forming units (CFUs) were contained in 50  $\mu$ l of the diluted samples. The bacteria were diluted with PBS (pH 7.4) in a series of 10-fold dilutions from 10<sup>4</sup> to 10<sup>1</sup> bacteria in 2  $\mu$ l. The IS6110 DNA target was diluted with distilled water from 10<sup>5</sup> to 10<sup>1</sup> molecules per 2  $\mu$ l. *M. bovis*, five NTM strains, *E. coli*,  $\beta$ -hemolytic *Streptococcus*, *S. aureus* and *S. pneumoniae* were used to evaluate the specificity of the HSAM assay. The target extraction and HSAM procedures were described above.

### Quality control

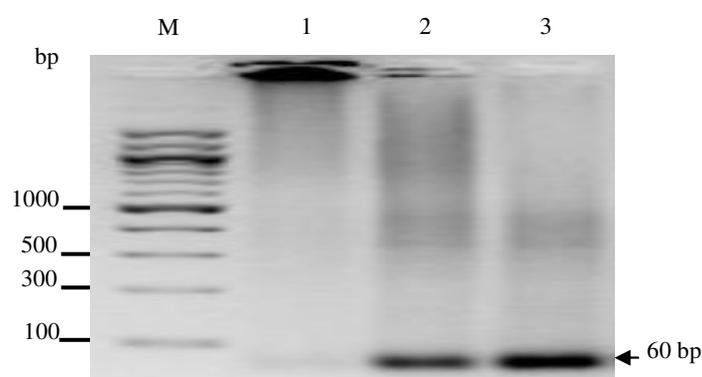
For the quality control of the HSAM assay, a fixed amount (about 10<sup>5</sup> CFU/ml) of MTB bacteria was spiked into a

normal sputum sample following the same sputum treatment procedures and DNA extraction protocols for clinical sputum samples. For each HSAM assay, positive and negative controls were included.

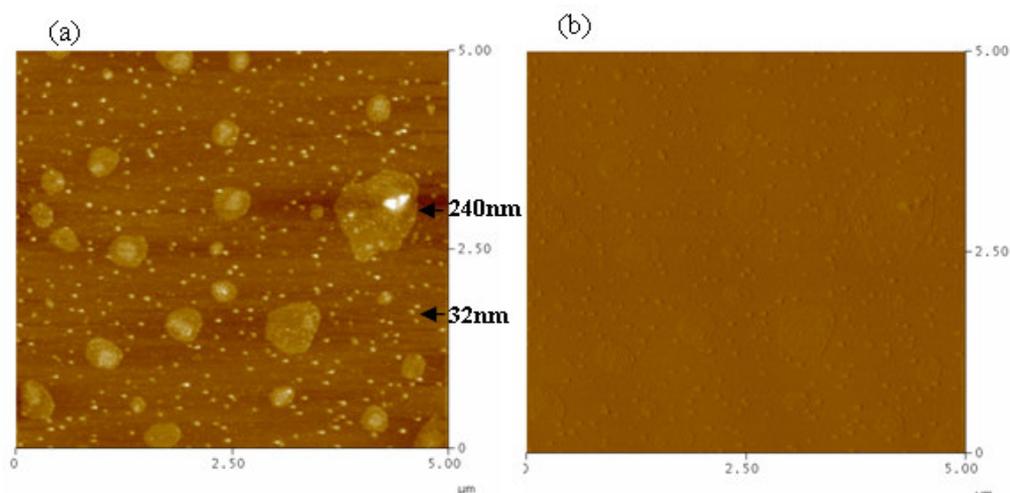
## RESULTS

To establish efficient conditions for nanoparticle formation, molar ratios of 2B-DNA to Poly-STV in the nanoparticle assembly were examined. As shown in Figure 2, a 1:5 ratio produced the maximum amount of nanoparticles that

are too large to migrate in a 1% agarose gel, which indicates the formation of nanoparticles (Lane 1). The ratio of 1:1 produced the lowest amount of nanoparticles, and free 2B-DNA is visible (Lane 3). The ratio of 1:2 is intermediate (Lane 2). Atomic force microscopy showed that the self-assembled nanoparticles are three-dimensional ellipsosomes, and the diameter and ramp diameter are 32 nm and 72 nm, respectively. Very few nanoparticles have a diameter of 240 nm, perhaps due to adhesion between the nanoparticles (Fig. 3).



**Figure 2.** Formation of the HSAM nanoparticles analyzed by 1% agarose gel electrophoresis. The selected molar ratios of 2B-DNA to Poly-STV were examined in the nanoparticle preparation. The ratio of 1:5 produced the maximum amount of nanoparticles. M: molecular marker, Lane 1 to 3: 1:5, 1:2 and 1:1, respectively.



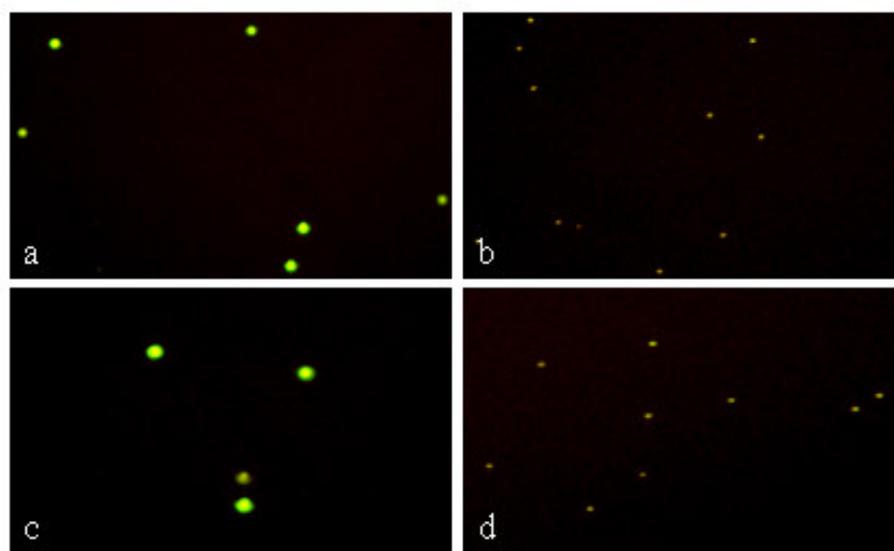
**Figure 3.** Representative atomic force microscopy images of self-assembled DNA-streptavidin nanoparticles show that the nanoparticles are three-dimensional ellipsosomes. They have a diameter of 32 nm and a ramp diameter of 72 nm. Very few nanoparticles have a diameter of 240 nm, which could be due to adhesion between nanoparticles. (a) Height image. (b) Phase image of the same sample area.

The analytical sensitivity of the HSAM assay was determined by testing serial dilutions of a synthetic *IS6110* DNA target and *M. tuberculosis* H37Rv. We initially determined the sensitivity of the HSAM assay using a ten-fold serial dilution of the synthetic *IS6110* DNA target. The lowest detection limit by the HSAM assay was 10 molecules (Fig. 4a), which was confirmed by observing the presence of green fluorescent beads. The assay sensitivity was further analyzed using the *M. tuberculosis* H37Rv strain. The results in Figure 4c indicate that the HSAM assay was able to detect as few as 10 bacteria.

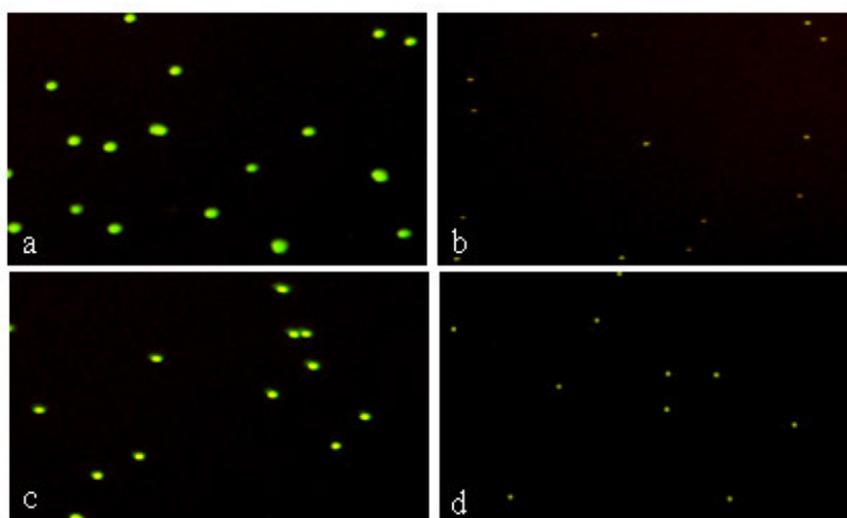
To determine the assay specificity, we tested the following 11 bacterial strains: *M. tuberculosis* H37Rv, *M. bovis*, five NTMs, *E. coli*, *S. aureus*,  $\beta$ -hemolytic *Streptococcus*, and *S. pneumoniae*. As expected, *M. tuberculosis* H37Rv and *M. bovis* were positive for the *IS6110* insertion sequence (Fig. 5 a,c), while the five NTMs

and other bacteria were negative (Fig. 5 b,d). These results confirmed the specificity of the HSAM assay.

A total of 176 clinical sputum specimens collected during the period August 2006 to March 2007 were diagnosed by traditional culture and biochemical identification methods. In parallel, the spent specimen sediments were subjected to DNA extraction and HSAM analysis, the results of which are summarized in Table 2. A total of 103 specimens were reported to contain MTBC strains through the standard culture and biochemical methods. The HSAM assay identified 97 specimens with MTBC strains, resulting in 88.3% sensitivity, 91.8% specificity, 93.8% positive predictive value and 84.8% negative predictive value. *IS6110* was identified in six specimens that were MTBC culture-negative. Among the 103 culture-positive MTBC specimens, 12 were *IS6110*-negative according to the HSAM assay.



**Figure 4.** Sensitivity of the HSAM assay for detecting the *IS6110* target. The HSAM reactions were initiated with synthetic *IS6110* targets consisting of  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ , and 10 copies or serial dilution of bacterial culture. The read-out of HSAM assay was carried out using fluorescent microscopy. (a) HSAM assay detected as few as 10 copies of synthetic *IS6110* targets as demonstrated by the presence of green fluorescent beads. (b) No green fluorescent beads in the absence of synthetic *IS6110* target. (c) Detection of as few as 10 bacteria *M. tuberculosis* H37Rv. (d) No green fluorescent beads using distilled water as negative control.



**Figure 5.** Specificity of the HSAM assay for detecting the IS6110 target in MTBC. Eleven bacteria were tested with the HSAM assay. The IS6110 insertion sequence was present only in MTBC. *M. tuberculosis* H37Rv and *M. bovis* were positive, while five NTMs and other bacteria were negative. (a) *M. tuberculosis* H37Rv; (b) *E. coli*; (c) *M. bovis*; (d) *M. kansasii*.

**Table 2.** Comparison of MTBC diagnosis results from 176 clinical sputum specimens by culture and HSAM assays.

| HSAM (no. of samples) | Culture <sup>a</sup> |          |
|-----------------------|----------------------|----------|
|                       | Positive             | Negative |
| Positive (97)         | 91                   | 6        |
| Negative (79)         | 12                   | 67       |
| Overall (176)         | 103                  | 73       |

<sup>a</sup>Culture and biochemical identification results served as the gold standard for diagnosis. Sensitivity: 88.3%, Specificity: 91.8%; positive predictive value: 93.8%, negative predictive value: 84.8%.

## DISCUSSION

Rapid detection of MTBC strains and their differentiation from NTMs are critical for initiating appropriate therapy and facilitating measures to prevent the dissemination of TB. A range of rapid tests based on nucleic acid amplification techniques have been developed for the direct detection of MTBC from clinical samples, including PCR. However, these methods may not satisfy the requirements for common use: high sensitivity and specificity, ease in application and cost-effectiveness (18). The relatively high cost of most molecular

methods may be one of the major characteristics that hinder the wide use of these systems, especially in developing countries where TB is more prevalent than in developed ones. The HSAM assay is a novel, simple detection method based on the self-assembly of DNA-STV nanoparticles. This assay is easy to perform, does not require any sophisticated detection equipment and is highly sensitive and specific. In addition, it is fast and inexpensive; it takes less than 5 h from the receipt of specimens to completion of the test.

The HSAM assay is a variation of traditional probe signal amplification schemes based on the concept of combining signal amplification by nanoparticles, magnetic bead-based DNA isolation, and fluorescence detection (13). In this study, we used a synthetic IS6110 DNA target and *M. tuberculosis* H37Rv to test the HSAM assay. The results obtained by testing ten-fold serial dilutions of the IS6110 target and *M. tuberculosis* H37Rv indicated an analytical sensitivity of 10 synthetic target molecules or 10 bacteria, respectively. Other researchers have reported a detection range of 1–100 cells for PCR techniques (8, 9, 20, 21). The increased sensitivity of the HSAM assay may be due to several factors. First, the three-

dimensional porous nanoparticles provide multiple binding sites for reporter molecules (biotinylated FITC), allowing for a thousand-fold amplification. The self-assembled nanoparticles are composed of bis-biotinylated single-stranded DNA (2B-DNA) and dextran-based poly-streptavidin (Poly-STV). The tetravalent binding capacity of STV, combined with its high affinity for biotin, lead to the formation of the three-dimensional nanoparticles (24). The nanoparticles differ from other DNA-streptavidin nanostructures containing double-stranded DNA and streptavidin that are widely used as reagents in immuno-PCR (10, 11) in which the 2B-DNA is used as a detection probe and is complementary to the DNA target sequence. Second, the HSAM assay detects *IS6110*, which is present at a level of approximately 10–16 copies per cell, which may enhance the sensitivity of the HSAM assay relative to others that detect target sequences with only a single or very low copy number. Third, Dynal magnetic beads were used as a solid support for the capture probe and as magnetic enrichment for target molecules, allowing the test to be performed directly from processed samples without the need for extensive nucleic acid purification.

While conventional culture and biochemical identification serve as the gold standards for diagnosis, we demonstrated that the HSAM assay is an excellent alternative for the detection of MTBC in 176 clinical sputum specimens. As a positive control, a fixed amount (about  $10^5$  CFU/ml) of MTB bacteria was spiked into a normal sputum sample, and distilled water was used as a negative control. Compared with conventional culture and biochemical diagnosis methods, HSAM resulted in 88.3% sensitivity, 91.8% specificity, 93.8% positive predictive value and 84.8% negative predictive value for MTBC diagnosis. The results are comparable to, or even better than, those obtained by PCR for MTBC diagnosis. The sensitivities for PCR range from 74% to 100% (8, 9, 20, 21), and acid fast bacilli (AFB) smear method sensitivities vary from 30% to more than 70% (1). Among the 103 cultures in which MTBC specimens were identified by culture and biochemical techniques, 91 were *IS6110*-positive in the HSAM

assay. In contrast, 12 specimens that were positive by traditional techniques were MTBC-negative in our assay, which may be due to too few bacterial cells in the sputum specimens. However, there were 6 culture-negative, *IS6110*-positive samples, which could be due to non-specific hybridization during the HSAM assay. The HSAM assay for MTBC detection can be performed anywhere, and there is no need for a contamination-proof hood or specific lab area, as is required for PCR. The HSAM assay can be used to detect different DNA and RNA targets, which is easily accomplished by altering the sequence of the capture and detection probes.

In summary, the present study showed that the HSAM assay can detect MTBC in clinical specimens. The method is highly sensitive and specific, economical, time-saving and simple. Although further adjustments are required to improve its sensitivity and quantitative aspects, we expect that the HSAM assay will be a useful tool for the rapid detection of MTBC.

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